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Barbara G. McClung 1/11/91
Barbara G. McClung Date

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PATENT

EXPRESSION AND USE OF HUMAN FIBROBLAST GROWTH FACTOR RECEPTOR

Description

Technical Field

This invention relates to the fields of molecular biology and pharmaceutical research. More specifically, this invention relates to the recombinant expression of a human high-affinity fibroblast growth factor (FGF) receptor, and its use in combination with glycosaminoglycans to model compounds capable of mimicking FGF binding.

Background of the Invention

The fibroblast growth factors (FGFs) are a family of structurally related polypeptides that regulate the growth and differentiation of a diverse number of cell types. Acidic and basic FGFs are mitogenic for cell types of mesenchymal, epithelial and neural origin (K. Thomas, FASEB J (1987) 1:434-440; D. Gospodarowicz, Meth Enzymol (1987) 147:106-119). The more recently discovered members of the FGF family have been implicated in early developmental processes and in epithelial cell growth and maintenance (R. Moore *et al*, EMBO J (1986) 5:919-924; A. Jakobovits *et al*, Proc Natl Acad Sci USA (1986) 83:7806-7810; P.W. Finch *et al*, Science (1989) 245:752-755). Currently, the FGF family consists of seven distinct gene

products including acidic and basic FGFs (M. Jaye *et al*, Science (1986) 233:541-545; J.A. Abraham *et al*, Science (1986) 233:545-548; J.A. Abraham *et al*, EMBO J (1986) 5:2523-2528), the product of the int-2 oncogene (R. Moore *et al*, *supra*; A. Jakobovits *et al*, *supra*), a growth factor identified from Kaposi's sarcoma DNA (hst-1 or KS-FGF) (P.D. Bovi *et al*, Cell (1987) 50:729-737; M. Taira *et al*, Proc Natl Acad Sci USA (1987) 84:2980-2984), FGF-5 (X. Zhan *et al*, Mol Cell Biol (1988) 8:3487-3495), FGF-6 (I. Marics *et al*, Oncogene (1989) 4:335-340) and keratinocyte growth factor, KGF or FGF-7 (P.W. Finch *et al*, *supra*).

The large number of FGFs and their diverse spectrum of activities suggests that several receptors may mediate their effects on cells. Indeed, for the acidic and basic FGFs themselves, two classes of receptors have been well documented which are distinguished by their affinities for FGF. For example, the binding of bFGF to a high affinity site on baby hamster kidney (BHK) cells occurs with a dissociation constant in the 20 pM range, whereas bFGF binding to the low affinity site occurs with a dissociation constant in the 2 nM range, and is released with 2M NaCl. The FGF receptor has been implicated as the entry portal for Herpes simplex virus (HSV). Several high affinity FGF receptor cDNAs have been cloned (P.L. Lee *et al*, Science (1989) 245:57-60; E. Pasquale & S.J. Singer, Proc Natl Acad Sci USA (1989) 86:5449-5453; M. Ruta *et al*, Oncogene (1988) 3:9-15; H.H. Reid *et al*, Proc Natl Acad Sci USA (1990) 87:1596-1600; A. Isacchi *et al*, Nuc Acids Res (1990) 18:1906; D.E. Johnson *et al*, Mol Cell Biol (1990) 10:4728-4736) and shown by structural homology to be members of the cell surface protein-tyrosine kinase family of proteins. This group of membrane-bound proteins are thought to play an important role in the regulation of cell growth. They include the receptors for

epidermal growth factor, platelet-derived growth factor, colony stimulating factor-1, insulin, and insulin-like growth factor-1 (for recent review see A. Ullrich & J. Schlessinger, Cell (1990) 61:203-212).

Structural analyses of the extracellular regions of the chicken FGF receptor cDNA suggests that the FGF receptors also belong to the immunoglobulin supergene family (P.L. Lee *et al*, *supra*). Accordingly, Reid *et al*, (*supra*) have found several forms of the bFGF receptor mRNA in developing mouse brain that contain either two or three immunoglobulin-like domains. Moreover, they detected a region of sequence variability between the first and second immunoglobulin-like domains. In this case, amino acids 148 and 149 are sometimes deleted in the predicted sequences for proteins that contain 2 immunoglobulin-like domains. Recently, four forms of the cDNA encoding the human two immunoglobulin-like domain FGF receptor have been identified (D.E. Johnson *et al*, *supra*). Two of these forms are homologous to the mouse two immunoglobulin-like domain FGF receptor in that they vary at amino acids 148 and 149 (H.H. Reid *et al*, *supra*). While the other two forms of the human FGF receptor also vary at these amino acids, they are unique in that they lack a transmembrane domain and the cytoplasmic tyrosine kinase domain. More recently, a fifth form of the human FGF receptor cDNA has also been isolated (A. Isacchi *et al*, *supra*), and is homologous to the mouse three immunoglobulinlike-domain FGF receptor. In addition to the five forms of the FGF receptor, Southern blot analysis and the cloning of two related cDNAs, bek (H.H. Reid *et al*, *supra*; S. Kornbluth *et al*, Mol Cell Biol (1988) 8:5541-5544) and a bek-related molecule (H.H. Reid *et al*, *supra*), indicate that FGF receptors are members of a multigene family.

A number of researchers have recently reported expression of various FGF receptors. See R.J. Kaner *et al*, Science (1990) 248:1410-13; A. Mansukhani *et al*, Proc Nat Acad Sci USA (1990) 87:4378-82; C.A. Dionne *et al*, EMBO J (1990) 9:2685-92; and D.P. Mirda & L.T Williams, Clin Res (1990) 38:310A. However, the reported experiments in general do not disclose the expression of human FGF receptor in quantity sufficient for study.

In order to usefully study the binding of FGF analogs to the FGF receptor, one must have available sufficient quantities of active receptor for study. Further, the receptor must be in a useful form.

Disclosure of the Invention

A new human FGF receptor has now been cloned and expressed using cDNA obtained from a human liver cell line. The expression of high levels of the extracellular region of this FGF receptor in a baculovirus/insect cell system yields a high affinity FGF-binding protein that is active in radioreceptor assays, inhibits cell growth and that can be used to study the ligand-receptor interaction. Furthermore, four forms of the cDNAs that encode the FGF receptor have now been identified in several tissues and cell lines, suggesting there exists an extensive distribution of alternate forms that are generated by differential RNA splicing.

Thus, one aspect of the invention is a recombinant FGF receptor (rFGF-R), which is capable of binding aFGF and/or bFGF. Another aspect of the invention is a recombinant fragment of FGF-R comprising the extracellular domain (soluble FGF-R, or "sFGF-R"), which is capable of binding aFGF and/or bFGF.

Another aspect of the invention is a method for detecting FGF in a sample, by employing rFGF-R in a manner analogous to an anti-FGF antibody in any form of immunoassay. For example, one may detect FGF by providing a support comprising rFGF-R bound to a solid surface, contacting the support with a sample to be assayed for FGF, removing the portion of the sample which does not bind to the support, and detecting the presence of bound FGF on the support (*e.g.*, by using a labeled anti-FGF antibody, by competition with labeled FGF, *etc.*).

Another aspect of the invention is a method for inhibiting the activity of FGF, using rFGF-R. Thus, rFGF-R may be used to inhibit FGF-mediated activities. For example, one method of the invention is the inhibition of FGF-dependent tumor growth by administering an effective amount of sFGF-R. Another method of the invention is the method of inhibiting angiogenesis (*e.g.*, of a tumor) by administering an effective amount of sFGF-R. Another method of the invention is the method of inhibiting FGF-dependent cell growth *in vitro* by administering rFGF-R.

Another aspect of the invention is the use of rFGF-R to screen and identify compounds which mimic FGF binding. Compounds identified in this manner may be agonists or antagonists. Agonists are useful in situations in which FGF activity is beneficial, *e.g.*, for acceleration of wound healing, nerve outgrowth, and the like. Antagonists are useful for inhibiting the activity of FGF, for example, to inhibit the growth of FGF-dependent malignancies, and the like. Compounds may be screened by providing a support having bound rFGF-R, contacting the support with a candidate compound, and detecting any compound bound to the support. Suitable compounds may also be used to block or inhibit binding by Herpes virus.

Brief Description of the Drawings

Figure 1 depicts a schematic diagram of the human FGF receptor cDNA (*flg* 5) and sequencing strategy. The translated regions are boxed, and various shaded domains are indicated: S, signal peptide; 1-3, immunoglobulinlike-domains 1-3; ARR, acidic amino acid rich region; TM, transmembrane region; TK, tyrosine kinase domains. Potential Asn-linked glycosylation sites are also indicated (♦) as are the BglII (G) and EcoRI (E) restriction endonuclease sites. Although shown, the location of the most carboxyl-terminal consensus glycosylation site most likely precludes its use. Sequences were obtained by using M13 primers and specific internal primers. Arrows indicate the direction and extent of individual sequencing runs. The DNA sequence is in the Genbank and EMBL data bases, and accession numbers are available from these organizations.

Figure 2 depicts an amino acid sequence comparison of the six different human FGF receptor forms. Sequences have been aligned for maximum identity and those that differ or are deleted have been boxed. Various domains (abbreviations as in Fig. 1) and regions used for PCR primers (P1-P4) are indicated above sequence 1 (*flg* 5). The putative signal peptidase cleavage site is also indicated (↓). Sequence 2 was from A. Isacchi *et al*, *supra* and sequences 3-6 were from D.E. Johnson *et al*, *supra*.

Modes of Carrying Out The Invention

A. Definitions

The term "FGF receptor" or "FGF-R" as used herein refers to the human FGF receptor or a fragment thereof capable of binding FGF in the presence of heparin, and having an amino acid sequence substantially as depicted in Figure 2. The term "rFGF-R" refers to active FGF-R prepared by recombinant means.

A preferred form of rFGF-R is soluble rFGF-R ("sFGF-R"), which is a truncated form obtained by expressing only the extracellular domain. It is surprisingly found that the truncated form retains its FGF-binding activity, and thus may be used to assay compounds for FGF-like binding activity or to bind actual FGF and thus inhibit its activity. The preferred sFGF-R of the invention is a 58 kDa glycoprotein which binds bFGF with a K_d of 2-5 nM.

The term "substantially pure" indicates a protein or composition that is essentially free of contaminants similar to the protein. In the present case, the normal contaminants associated with FGF-R predominately include human proteins. Thus, rFGF-R is substantially pure if it is essentially free of human proteins. "Essentially free" is determined by weight. In general, a composition containing 70% rFGF-R and $\leq$ 30% human proteins may be considered substantially pure. Preferably, the composition will be at least 80% rFGF-R, more preferably at least 90%, and most preferably $\geq$ 95% rFGF-R. The presence of dissimilar components does not affect the determination of purity, thus a composition containing 0.7 mg/mL rFGF-R in PBS will still be considered substantially pure if it contains $\leq$ 0.3 mg/mL other human proteins.

The term "effective amount" refers to an amount of rFGF-R sufficient to exhibit a detectable therapeutic effect. The therapeutic effect may include, for example, without limitation, inhibiting the growth of undesired tissue or malignant cells, inhibiting the growth of FGF-dependent cells in the presence of cells not so constrained, inhibiting infection by HSV, and the like. The precise effective amount for a subject will depend upon the subject's size and health, the nature and severity of the condition to be treated, and the like. Thus, it is not possible to specify an exact effective

amount in advance. However, the effective amount for a given situation can be determined by routine experimentation based on the information provided herein.

The term "specific binding" indicates binding which defines a generally stoichiometric ligand-receptor relationship. Specific binding indicates a binding interaction having a low dissociation constant, which distinguishes specific binding from non-specific (background) binding.

"PCR" refers to the technique of polymerase chain reaction as described in Saiki, *et al.*, Nature 324:163 (1986); U.S. Patent No. 4,683,195; and U.S. Patent No. 4,683,202.

B. General Method

The FGF-R may be cloned and expressed as described below, based on the disclosed PCR primer sequences. It is presently preferred to express rFGF-R using a baculovirus vector, see, e.g., commercially available kits from Invitrogen, San Diego CA ("MaxBac" kit), Summers and Smith, Texas Agricultural Experiment Station Bulletin No. 1555 (1987). Although other expression systems are not excluded, see, e.g., Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor NY, 1989 (bacterial expression); Barr *et al.*, Yeast Genetic Engineering, Butterworths, Boston, MA, 1989 (yeast expression); U.S. Patent Nos. 4,399,216; 4,912,040; 4,740,461; 4,959,455 (these patents are incorporated herein by reference) (mammalian cell expression).

Using a baculovirus expression system, the protein is expressed as a glycoprotein in insect cells, and may easily be purified using lentil lectin chromatography. Active truncated forms of rFGF-R may be prepared by expressing only the extracellular binding domain, preferably aa_{1-374}.

Immunoassay protocols may be based, for example, upon competition, direct reaction, or sandwich type assays. Protocols may also use solid supports, or may involve immunoprecipitation. Most assays involve the use of labeled antibody or ligand. The labels may be, for example, fluorescent, chemiluminescent, radioactive, dye molecules, or enzymes. Assays that amplify the signals from the probe are also known, for example, assays that utilize biotin and avidin, and enzyme-labeled and mediated immunoassays, such as ELISA assays.

Typically, an assay for detecting FGF or FGF analogs will involve selecting and preparing the test sample, such as a biological sample, and then incubating it with the FGF-R under conditions that allow receptor-ligand complexes to form. Such conditions are well known in the art. In a heterogeneous format, the receptor is bound to a solid support to facilitate separation of the sample from the receptor after incubation. Examples of solid supports that can be used are nitrocellulose, in membrane or microtiter well form; polyvinylchloride, in sheets or microtiter wells; polystyrene latex, in beads or microtiter plates; polyvinylidine fluoride, known as Immobulon™; diazotized paper; nylon membranes; activated beads; and Protein A beads. The solid support is typically washed after separating it from the test sample. In a homogeneous format, the test sample is incubated with a soluble form of the receptor in solution (e.g., sFGF-R), under conditions that will precipitate any receptor-ligand complexes that are formed, as is practiced in the art. The precipitated complexes are then separated from the test sample, for example, by centrifugation.

The complexes formed comprising FGF or FGF analogs in either the homogenous or heterogenous format can be detected by

any of a number of techniques. Depending on the format, the complexes can be detected with labeled antibodies against FGF-receptor, FGF, or FGF analogs; or labeled FGF-R or, if a competitive format is used, by measuring the amount of bound, labeled competing FGF or FGF analogs.

The use of enzyme-linked antibodies is one well-known method for detecting receptor-ligand complexes. This method depends upon conjugation of an enzyme to antibodies against FGF, FGF analogs, or FGF-R, and uses the bound enzyme activity as a quantitative label. Enzymes suitable for labeling are known in the art, and include, for example, horseradish peroxidase and urease. Enzyme activity, bound to the receptor-ligand complex, is measured by adding the specific enzyme substrate, and determining product formation or substrate utilization. For ease, the substrate can be chosen so that substrate utilization can be determined colorimetrically.

Kits suitable for FGF or FGF analog detection can contain the appropriate reagents, which may or may not be labeled, such as FGF-R, FGF, or FGF analogs, or antibodies directed against FGF-R, FGF, or FGF analogs in suitable containers; along with the remaining reagents and materials required for the conduct of the assay (e.g., wash buffers, detection means, such as labeled FGF or FGF analogs or labeled anti-FGF-R), as well as a suitable set of assay instructions.

It is convenient to use sFGF-R to assay compounds for FGF-like binding activity, and thus to identify compounds which may serve as agonists or antagonists. In a typical screening assay, sFGF-R is adsorbed onto a support (such as the wells of a microtiter plate), fixing with glutaraldehyde if necessary. Alternatively, the sFGF-R may be immobilized using a lectin, such as ConA. The support is then contacted with a solution containing the compound(s) in question, allowed to incubate,

and the remaining solution removed. After several washes, the plate is examined for the presence of bound compound. Bound compound may be detected by spectroscopic means (for example colorimetric or fluorometric means, depending on the characteristics of the compound), or by radioactive means if the compound has been so labeled. Alternatively, one may assay the compound for competition with labeled FGF. A large number of such assays can be performed and analyzed simultaneously, for example by conducting the experiments in an array (e.g., using a microtiter dish). In order to more completely model FGF, the compounds should be assayed for binding in the presence of heparin. It is theorized that both low affinity and high affinity FGF receptors are required for full FGF activity *in vivo*. It has now been found that FGF fails to bind the high affinity receptor with the same affinity in the absence of the low affinity receptor, but that the presence of sufficient heparin restores binding. Thus, one may completely model the FGF binding system *in vitro* using only sFGF-R and heparin. Compounds which exhibit a high affinity for sFGF-R may then be assayed for biological activity against FGF-R, or for inhibition of HSV infectivity, in an appropriate whole cell assay.

FGF is known to stimulate the growth and proliferation of many cell types, including normal cells of mesenchymal, epithelial or neural origin, and tumor cells, including melanoma. Some tumor types depend upon autocrine activity of FGF for proliferation. Accordingly, it is possible to employ rFGF-R to inhibit such proliferation *in vivo* or *in vitro*. *In vivo*, one may administer an effective amount of rFGF-R, preferably sFGF-R, to inhibit the undesirable growth of normal tissue (e.g., in scar formation, psoriasis, and other hyperplasias) or malignant tissue (as in the case of tumors,

carcinomas, and the like). As FGF may stimulate angiogenesis, administration of rFGF-R may be used, for example, to inhibit the vascularization of inoperable tumors.

HSV is believed to invade susceptible cells through the FGF receptor. Thus, one may inhibit HSV infection by administering sFGF-R to susceptible surfaces, for example the mucosal membranes. Such administration is preferably in the form of a lotion, ointment, salve, or aerosol.

Compositions of the invention for administration will generally include an effective amount of sFGF-R in addition to a pharmaceutically acceptable excipient. Suitable excipients include most carriers approved for oral or parenteral administration, including water, saline, Ringer's solution, Hank's solution, and solutions of glucose, lactose, dextrose, ethanol, glycerol, albumin, and the like. These compositions may optionally include stabilizers, antioxidants, antimicrobials, preservatives, buffering agents, surfactants, and other accessory additives. A presently preferred vehicle comprises about 1 mg/mL serum albumin in phosphate-buffered saline (PBS). A thorough discussion of suitable vehicles for parenteral administration may be found in E.W. Martin, "Remington's Pharmaceutical Sciences" (Mack Pub. Co., current edition).

The precise dosage necessary will vary with the age, size, and condition of the subject, the nature and severity of the disorder to be treated, and the like: thus, a precise effective amount cannot be specified in advance. However, appropriate amounts may be determined by routine experimentation with animal models. In general terms, an effective dose sFGF-R will range from about 10 μg/Kg to about 5 mg/Kg.

C. Examples

The examples presented below are provided as a further guide to the practitioner of ordinary skill in the art, and are not to be construed as limiting the invention in any way.

Example 1

(Procedures)

Materials:

Human basic FGF was produced in yeast, as described by P.J. Barr *et al*, J Biol Chem (1988) 263:16471-16478. Enzymes for molecular biology were obtained from Boehringer Mannheim, New England Biolabs and Pharmacia. The λZAP cDNA cloning kit was obtained from Stratagene. The PCR amplification kit was from Perkin Elmer Cetus. Radiochemicals were obtained from Amersham or New England Nuclear. Lentil lectin Sepharose® 4B and methyl-α-D-mannopyranoside were obtained from Sigma. Human liver poly $(A)^+$ RNA was obtained from Clontech (Palo Alto, CA) and human osteosarcoma tissue was a gift from Dr. Marshall Urist (University of California, Los Angeles).

Hep G2 (ATCC No. HB 8065), a human hepatoma cell line; 293, a human embryonic kidney cell line (ATCC No. CRL 1573); and *Spodoptera frugiperda* clone 9 (Sf9) an insect cell line, were obtained from the American Type Culture Collection (Rockville, MD). Hep G2 and 293 cells were grown to subconfluency in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, 100 U/mL penicillin and 100 mg/mL streptomycin at 37°C in 5% CO_2. Sf9 cells were adapted to grow in Excell-400 serum free medium (J.R. Scientific). Procedures for culturing and subculturing the cells, transfections and production of high titer viral stocks were

performed as described (M.D. Summers & G.E. Smith, (1987) A Manual of Methods of Baculovirus Vectors and Insect Cell Culture Procedures, Texas Agriculture Experiment Station Bulletin No. 1555). Wild type *Autographa californica* nuclear polyhedrosis virus (AcMNPV) viral DNA and transfer plasmid pAc373 were a gift of Dr. Max Summers (Texas A&M University).

Example 2

(Expression of EC-FGF Receptor)

Oligonucleotide Synthesis:

Oligonucleotide adapters, probes and sequencing primers were synthesized by the phosphoramidite method using Applied Biosystems (Foster City, CA) model 380A and 380B synthesizers, purified by polyacrylamide gel electrophoresis and desalted on SEP-PAK C_{18} cartridges (Waters, Milford, MA). The oligonucleotide probes used for screening the cDNA library were complementary to nucleotides 1-30 (5'-A-TAACGGACCTTGTAGCCTCCAATTCTGTG-3') and nucleotides 1840-1869 (5'-GCGGCGTTTGAGTCCGCCATTGGCAAGCTG-3') of the published *flg* nucleic acid sequence (M. Ruta *et al*, *supra*). The two PCR primers used to amplify the extracellular region of the FGF receptor (*flg*5) cDNA consisted of a sense primer, P4 (5'-CCAACCTCTAGAGGATCCACTGGGATGTGGAGCTGGAAGTGC-3') containing the ribosome binding site plus amino acids 1-6 of *flg* 5 and an antisense primer, P3 (5'-GTAAGCGGCCGCGGATCCTTACTACTCCAGGTACAGG-GGCGA-3') containing amino acids 369-374 of *flg*5 and directly followed by a termination codon. Both primers contain BamHI sites to facilitate cloning into pAc373. Two additional PCR primers were used to identify two and three immunoglobulinlike domain FGF receptors in various tissues. They were a sense primer, P1 (5'-CCATTTGGATCCGTCACAGCCACACTCTGCACCGCT-3') encoding amino acids 14 to 21 of *flg* 5 and an antisense primer

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P2 (5'-CCATTTGTCGACTTCCATCTTTTCTGGGGATGTCCA-3') encoding the complement of amino acids 154 to 161 of *flg* 5. The primers contain BamH1 and SalI sites to facilitate cloning into M13 sequencing plasmids.

RNA Isolation and Construction and Screening of the cDNA Library:

RNA was isolated by the guanidinium thiocyanate method (J.M. Chirgwin *et al*, Biochem (1979) 18:5294-5299) with modifications (G.J. Freeman *et al*, Proc Natl Acad Sci USA (1983) 80:4094-4098). Poly(A)$^+$ RNA was purified by a single fractionation over oligo(dT) cellulose (H. Aviv & P. Leder, Proc Natl Acad Sci USA (1972) 69:1408-1412). The construction and screening of the Hep G2 library in λZAP has been described (J. Zapf *et al*, J Biol Chem (1990) 265:14892-14898). The probes were labeled with T_4 polynucleotide kinase and [γ-^{32}P]-ATP (J. Sambrook *et al*, (1989) Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), 2nd Ed) to a specific activity of 1-2 x 10^8 cpm/mg. Approximately 600,000 recombinant phages from the Hep G2 cDNA library were screened on duplicate nitrocellulose filters (Millipore, HATF 137), with two *flg* oligonucleotide probes. Areas of plaques that hybridized to both probes were further purified.

Plasmid Isolation, Subcloning and Sequencing:

Bluescript SK(-) plasmids containing the putative *flg* cDNA inserts were released from λZAP by the M13 rescue/excision protocol described by the supplier (Stratagene). Plasmid DNA was isolated by the alkaline lysis method (J. Sambrook *et al*, *supra*). The cDNA inserts containing the putative *flg* sequence were excised from the Bluescript SK(-) vector by BglII or EcoRI digestion and fractionated by agarose gel electrophoresis. Inserts were excised from the gel and passively eluted for 16 h

with gentle shaking in 10 mM Tris-hydrochloride, pH 7.5, 1 mM EDTA (TE), purified on elutip-D columns (Schleicher and Schuell) and subcloned into M13 sequencing vectors (C. Yanisch-Perron *et al*, Gene (1985) 33:103-119). PCR-amplified DNA was similarly purified. DNA sequencing was performed by the dideoxy chain termination method (F. Sanger *et al*, Proc Natl Acad Sci USA (1977) 74:5463-5467) using M13 primers as well as specific internal primers. Ambiguous regions were resolved using 7-deaza-2'-deoxyguanosine-5'-triphosphate (P.J. Barr *et al*, Biotechniques (1986) 4:428-432) and Sequenase (US Biochemicals).

To isolate full length FGF receptor encoded cDNAs, 600,000 recombinants from a λZAP-human hepatoma cell line (Hep G2) cDNA library were screened with oligonucleotide probes derived from the 5'- and 3'-ends of a partial *flg* cDNA (M. Ruta *et al, supra*). Six clones were identified that hybridized to both probes. BglII restriction endonuclease digestion of the cDNA inserts and gel analysis suggested that three of the six clones contained the complete coding sequence. Four BglII fragments of 1.6, 1.1, 0.6, and 0.55 Kb and two EcoRI fragments of 2.7 and 1.2 Kb were identified in the longest cDNA clone, *flg* 5 (Fig. 1). BglII and EcoRI sites are also present in the flanking adapters that were used to make the cDNA library. The BglII and EcoRI fragments of *flg* 5 cDNA were isolated, cloned into M13 mp19 and sequenced. A detailed sequencing strategy is shown in Fig 1. The *flg* 5 cDNA encodes a protein of 820 amino acids and is flanked by 671 and 753 nucleotides of 5'- and 3'-untranslated regions, respectively. The encoded protein revealed a structure that included a signal peptide, three extracellular immunoglobulinlike domains, an acidic amino acid-rich region, a transmembrane domain and a split intracellular tyrosine kinase domain. These domains have been identified

previously on the chicken (P.L. Lee *et al, supra*), the mouse (H.H. Reid *et al, supra*) and most recently, several human FGF receptors deduced from cDNA sequences (A. Isacchi *et al, supra*; D.E. Johnson *et al, supra*). The encoded receptor also contains eight consensus N-linked glycosylation sites in the extracellular region and one in the cytoplasmic tyrosine kinase domain.

The amino acid sequence encoded by *flg* 5 cDNA is shown in Fig. 2 (top row). For comparison, five other previously identified forms of the human FGF receptors are shown (A. Isacchi *et al, supra*; D.E. Johnson *et al, supra*) and are aligned for maximum amino acid sequence identity. The identified structural domains are indicated above the *flg* 5 sequence, as are regions corresponding to the PCR primers. The putative signal peptidase cleavage site (G. von Heijne, Nuc Acids Res (1986) 14:4683-4690) after Ala_{21} is indicated (↓). Differences or deletions of amino acids are boxed. The three most notable differences between the six FGF receptors are: i) a large deletion near the N-terminus in FGF receptors 3-6 (aa_{31-119}) that spans the entire first immunoglobulinlike domain; ii) truncation of receptors 5 and 6, which differ from the other FGF receptors in their carboxyl terminal amino acids ($aa_{221-300}$ and $aa_{223-302}$ respectively), with consequent deletion of their transmembrane and cytoplasmic domains; and iii) deletion of amino acids 148 and 149 in FGF receptors 1, 3 and 5. Other differences in FGF receptor-3 (aa_{101}) and FGF receptor-2 (aa_{817}) are also noted. The partial *flg* sequence (15) is not shown, but has an N-terminal amino acid corresponding to position 198 of FGF receptor-1. Accordingly, it may be encoded by the cDNAs of FGF receptors 1, 2, 3 or 4. It is important to note however, that the *flg* sequence displays a difference from FGF receptors 1-4 in the tyrosine kinase

domain at $aa_{670-674}$, due to three nucleic acid deletions flanking this region that results in a limited frame shift.

PCR Amplification:

Amplification reactions were performed according to the supplier of the PCR kit (Perkin Elmer Cetus). PCR primers and template were at a final concentration of 1 mM and 0.1-0.5 mg/mL, respectively. The cDNA encoding *flg*5 was used as a template DNA for the construction of EC-FGF receptor in pAc373. For expression studies, template DNA was reverse transcribed from mRNA as described (J. Zapf *et al*, *supra*). 30 cycles of PCR were performed using a Perkin Elmer Cetus DNA thermal cycler. Each cycle consisted of a 94°C, 1 min denaturation step; a 55°C, 2 min annealing step; and a 72°C, 3 min extension step. The extension step in the last cycle was 7 min.

Construction of Recombinant EC-FGF Receptor Virus:

The PCR amplified DNA fragment encoding the extracellular domain of the FGF receptor was digested with BamH1, gel purified and ligated to BamH1 cut, calf intestinal phosphatase-treated pAc373. Recombinant plasmids were analyzed for EC-FGF receptor cDNAs inserted in the correct orientation by restriction endonuclease digestion and agarose gel electrophoresis.

The recombinant plasmid was cotransfected with wild-type AcMNPV viral DNA into Sf9 cells by the calcium phosphate transfection procedure (M.D. Summers & G.E. Smith, *supra*). Recombinant viruses were identified in the first round of plaque screening by hybridization with *flg* 5 cDNA that was ^{32}P-labeled by replacement synthesis (J. Sambrook *et al*, *supra*). The recombinant viruses were further purified by visual screening for the occlusion negative phenotype in two additional rounds.

The recombinant baculovirus that expressed EC-FGF receptor was constructed by ligating PCR-amplified DNA encoding amino acids 1-374 of the *flg* 5 cDNA into the BamH1 site of the baculovirus transfer vector pAc373. The PCR primers contained flanking BamH1 sites to facilitate cloning. In addition, the 5' sense primer (P4) contained, directly upstream from the initiation codon, the -1 to -5 nucleotides of the *flg* 5 cDNA sequence that are implicated in ribosome binding (M. Kozak, Nuc Acids Res (1984) 12:857-87239). The 3'-antisense primer (P3) contained two termination codons TAG and TAA directly after amino acid 374. Co-transfection of Sf9 cells with AcMNPV viral DNA and the recombinant construct (pAc373-EC-FGF receptor) by the calcium phosphate method (M.D. Summers & G.E. Smith, *supra*) generated recombinant baculovirus that were subsequently purified by plaque hybridization and visual screening.

Expression and Purification of EC-FGF Receptor:

Sf9 cells were seeded in T-150 flasks at 5×10^7 cells/flask for small scale production of EC-FGF receptor. After 2 hr, the cells were infected with recombinant virus and incubated for 68-72 hrs. For larger scale production of EC-FGF receptor, Sf9 cells were infected with recombinant virus, incubated for 1 hr at 25°C, and then incubated in 3L spinner flasks at 3×10^6 cells/ml for 72-97 hrs. The conditioned medium from the above cultures were centrifuged for 30 min. at 14,000 xg at 4°C to partially clarify the recombinant virus. An aliquot of the supernatant was analyzed for EC-FGF receptor by 15% trichloroacetic acid precipitation, denaturing SDS-polyacrylamide gel electrophoresis (PAGE) (U.K. Laemmli, Nature (1970) 227:680-685) and visualization by Coomassie blue staining.

To further purify the EC-FGF receptor, the clarified supernatant was adjusted to 25 mM Hepes, pH 7.3, and loaded

onto a lentil lectin Sepharose® 4B column equilibrated with 150 mM NaCl, 25 mM Hepes, pH 7.3, 1 mM $CaCl_2$ and 1 mM $MnCl_2$. The column was washed in this equilibration buffer until no protein could be detected (OD_{280} ≈0) in the flow-through. The EC-FGF receptor was then eluted with 10% methyl-α-D-mannopyranoside, 25 mM Hepes, pH 7.3. Peak fractions were pooled, concentrated (Centricon 30) and stored in 10 mM Tris, pH 7.0, at -80°C. Aliquots from the various stages of purification were analyzed by SDS-PAGE (U.K. Laemmli, *supra*) and visualized by Coomassie blue staining.

To analyze EC-FGF receptor expression by the recombinant EC-FGF receptor-containing baculoviruses, Sf9 cells were infected with either wild type AcMNPV or EC-FGF receptor-AcMNPV. After 68 hours of incubation, proteins in the supernatant were precipitated and analyzed by SDS-PAGE and Coomassie blue staining. The resulting gel showed that the most intensely stained protein band in the supernatant (M_r = 58,000) is present only in the EC-FGF receptor-AcMNPV-infected cells and is not in the AcMNPV-infected cells, suggesting that this protein is the EC-FGF receptor. Six recombinant EC-FGF receptor-containing baculoviruses were analyzed for EC-FGF receptor expression in Sf9 cells. The level of EC-FGF receptor expression was essentially identical.

Analysis of EC-FGF Receptor Oligosaccharides:

Oligosaccharides contained in the purified EC-FGF receptor were analyzed by endoglycolytic cleavage with N-glycanase (Genzyme, Boston) according to the supplier's specifications. The products were analyzed by SDS-PAGE (U.K. Laemmli, *supra*) and visualized by Coomassie blue staining. The expected M_r for an unmodified EC-FGF receptor is ~40,000, suggesting that post-translational modification of the receptor occurs in insect cells. There are eight potential N-

glycosylation sites in the extracellular region of the FGF receptor to which oligosaccharides may be attached. To determine if N-linked oligosaccharides were present and contributed to the apparent M_r of the EC-FGF, the molecule was digested with N-glycanase. Digestion of EC-FGF receptor reduced the apparent M_r from 58,000 to 52,000, indicating that oligosaccharides were attached to the receptor through asparagine residues. In further support of this result, the EC-FGF receptor was purified by lentil lectin affinity chromatography.

Example 3

(FGF Receptor Binding and Activity Assays)

Radioreceptor assay:

The effects of the EC-FGF receptor on the binding of radioiodinated basic FGF to its receptor was examined using a radioreceptor assay as described in the art. Briefly, baby hamster kidney cells were maintained in Hepes (25 mM) buffered DMEM supplemented with 5% calf serum and antibiotics and were grown to sub-confluence in 24-well dishes. The cells were washed twice with phosphate buffered saline and incubated for 3 hours at 4°C with the indicated concentrations of the peptides and 1 ng (100,000 cpm) of labelled basic FGF in 300 μL of DMEM containing 0.1% gelatin. The medium was aspirated and the cells washed twice with 0.5 mL PBS and twice with 0.5 mL of PBS containing 2 M NaCl. The amount of ^{125}I-FGF bound to the high affinity receptor was determined by quantitating the amount of radioactivity in the cell lysate obtained with 0.1% Triton® X-100 in PBS, pH 8.4.

Mitogenesis assay:

The effects of the peptides on mitogenesis was determined using Swiss 3T3 fibroblasts as described. Briefly,

cells were plated at a concentration of 20,000 cells/well in 96 microwells and grown for two days in Hepes (25 mM) buffered DMEM containing 10% fetal calf serum and antibiotics. On the third day, the cells were washed twice with DMEM with no additives and the cells synchronized by a further incubation for two days in 0.5% fetal calf serum. At the time of assay, the test substances (basic FGF, EC-FGFR or both together) were added directly to the cells in 10 μL of DMEM supplemented with 0.1% BSA. Eighteen hours later, 1 μCi of ^{3}H-thymidine was added to the cells, and 24 hours after the addition of the peptides, the media was aspirated, the cells washed with PBS and the proteins precipitated with 50% trichloroacetic acid. After three washes, the cells were solubilized overnight with 1 N NaOH and the amount of radioactivity incorporated into DNA was determined by scintillation counting.

Cell Proliferation Assays:

The EC-FGF receptor was tested for its ability to inhibit basic FGF stimulated adrenal capillary endothelial (ACE) cell proliferation. Aliquots of receptor preparation were added to ACE cells and four days later, the cell number was established using a Coulter particle counter. For comparison purposes, 2 ng/ml of recombinant human basic FGF increased cell proliferation from 27,500 ± 2,100 cells/well to 133,300 ± 1,800 cells/well.

Receptor dependent tyrosine phosphorylation:

Swiss 3T3 cells were treated at 37°C for 5 minutes with no additives or with basic FGF (15 ng/mL), EC-FGF receptor (10 mg/mL) or basic FGF (15 ng/mL) and EC-FGF (10 mg/mL) added together. The cells were then harvested in a 2.5× Laemmli's buffer, the proteins separated on 8% polyacrylamide SDS-PAGE gels and the presence of tyrosine phosphorylated proteins

examined by Western blotting with a specific anti-phosphotyrosine antibody.

The FGF binding properties of EC-FGF receptor was determined using a soluble binding assay (adapted from the assay described by J.E. Robinson *et al*, J Immunol Meth (1990) 132:63-71). EC-FGF receptor, attached to concanavalin A coated plastic wells, was incubated with ^{125}I-bFGF and increasing concentrations of bFGF. Scatchard analysis of ^{125}I-FGF binding indicated a K_d of less than 5nM. An completely accurate K_d determination was not possible due to the non-specific binding of ^{125}I-FGF. Several blocking agents included in the assays, such as BSA, gelatin and heparan sulfate, were ineffective at blocking the non-specific binding of ^{125}I-FGF at low concentrations of ^{125}I-FGF.

The biological activity of the EC-FGF receptor was tested in several additional assay systems. First, the addition of EC-FGF receptor to endothelial cells in culture was shown to inhibit the proliferative effect of basic FGF. Because this cell type is known to synthesize basic FGF, it was suspected that the recombinant receptor might inhibit basal endothelial cell growth. As predicted, the expressed EC-FGF receptor can inhibit basal cell proliferation. Specificity of this effect was studied by incubating various cell types, that do not synthesize basic FGF, with the EC-FGF receptor. No effects were observed on BHK cells, A431 cells or on CHO cells. As expected, however, the addition of EC-FGF receptor to 3T3 cells inhibited the mitogenic response to basic FGF. Furthermore, it was observed that the EC-FGF receptor inhibited the growth of melanoma cells, a cell type previously shown to be dependent on the autocrine production of basic FGF.

To establish that the FGF/EC-FGF receptor complex did not recognize the basic FGF receptor, two experiments were

performed. First, the addition of the EC-FGF receptor preparation to BHK cells during the radioreceptor assay prevented the binding of ^{125}I-basic FGF to its receptor indicating that it binds basic FGF. The binding of ^{125}I-basic FGF to its low affinity receptor was also inhibited. Secondly, basic FGF fails to activate the tyrosine phosphorylation of either its cell membrane receptor or the characteristic 90-kDa substrate identified by Coughlin *et al*, J Biol Chem (1988) 263:988-993 when incubated in the presence of EC-FGF receptor.

Example 4

(Alternate Receptor Forms)

To determine whether multiple forms of the FGF receptor mRNAs are expressed in a single tissue or cell type, PCR was performed using mRNA isolated from human liver and osteosarcoma tissue as well as from the hepatoma cell line Hep G2 and the embryonic kidney cell line, 293. For these experiments, we used primers derived from the nucleic acid sequence encoding amino acids 14 to 21 and 154 to 160 of the *flg* 5 cDNA (P1 and P2, Fig. 2). These primers can detect either the two or three immunoglobulinlike-domain transcripts and should yield a 184 bp or 441 bp PCR-generated DNA product, respectively. Additionally, deletion variants at amino acid positions 148 and 149 can be readily identified by DNA sequence analysis of the PCR products. The truncated FGF receptors 5 and 6 shown (Fig. 2), are not distinguished by the primers selected.

Acrylamide gel analysis of the PCR products revealed DNA fragments of the expected size in all four tissues. DNA sequence analysis of the fragments revealed sequences that were identical, between the PCR primers, to the four forms of the FGF receptor shown in Fig. 2 (FGF receptor 1-4). Several

additional DNA fragments of approximately 280 bp and 550 bp were observed in all four PCR reactions. These PCR products were sequenced and shown to encode sequences unrelated to the FGF receptor. Thus, at least four forms of the FGF receptor are expressed in the tissues and cell lines examined. Taken together with the previous findings, these results indicate that multiple forms of FGF receptor mRNA are expressed in a wide variety of cell types and that as many as four forms of the receptor may be present on the surface of a cell type. Whether these forms are coexpressed in single cells remains to be determined.

Sequencing of the PCR fragments, identified an additional form of FGF receptor RNA that contained an intervening sequence. This form of the FGF receptor RNA most likely represents incompletely spliced heteronuclear RNA since a splicing event has already deleted the immunoglobulinlike 1 domain (aa_{31-119}). Interruption of the encoded amino acid sequence occurred at Pro_{150} (vertical lines A and C) and was separated by 248 nucleotides. This intervening region contains the dinucleotides GT and AG at its 5' and 3' ends, respectively, and is most likely derived from an intron.

The presence of an intron at aa_{150} suggested that an alternate splice donor site 2 amino acids upstream from 150 could generate the variant forms of the FGF receptor lacking amino acids 148 and 149. Indeed, six bases upstream (vertical line B) from amino acid 150, there is an acceptable splice donor site that could substitute for the downstream site and that would generate an in-frame deletion of amino acids 148 and 149. Thus, both the two and three immunoglobulinlike forms of the FGF receptor as well as the variant forms at amino acids 148 and 149 can be explained by alternate splicing.